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MORPHOMETRICS AND MORPHOLOGICAL CHARACTERIZATION OF ENTOMOPATHOGENIC NEMATODE ISOLATE TK1 FROM KENYA

Ngugi, CN[1]., Haukeland[3], S., Wachira, PM[2]., Mbaka, JN[1]. and Okoth.S[2].
[1]Kenya Agricultural and Livestock Research Organisation
[2]University of Nairobi
[3]International Center of Insect Physiology and Ecology

ABSTRACT

Entomopathogenic nematodes (EPNs), Steinernema and Heterorhabditis are insect parasitic nematodes used as biological agents for the management of economically important pests in agriculture. New EPN isolates can be characterised by morphology, morphometric characteristics and molecular analysis. The aim of this study was to describe an indigenous EPN isolate TK1 based on morphometric and morphological characteristics. The nematode was isolated from soils in an avocado orchard in Kenya Agricultural and Livestock Research Organisation (KALRO), Kandara. Insect baiting technique using last instar larvae of Galleria mellonella was used to establish and multiply the nematode isolate. Twenty 3rd stage infective juveniles (IJs) and 1stgeneration males of TK1 were heat killed and fixed. The measurements were expressed in % ratios and Means±SD ranges. The IJs were characterized by, body length of 834.54±87.36µm (658.60-986.89µm), short hyaline of 20.47±3.33µm (13.45-24.95µm) and tail length of 53.22±8.35µm (40.30-71.51) µm. The heat killed1st generation males were J shaped but strongly curved posteriorly almost spiral; excretory pore 106.37±13.77µm (84.51-140.13µm) and more posterior to the head; pharynx was short 119.39± 13.57µm (93.10-150.77µm). The spicule was 82.05±13.94µm (57.86-128.30µm) and gubernaculum was 45.28±5.89µm (34.48-54.87µm) about half the spicule length and a short tail (19.97±2.84µm (14.20-25.70) µm. Comparative analysis revealed relatedness to African glaseri Steinernematids S. karii, S. pwaniensis, S. ethiopense and

S. jeffreynse in IJs body length, c ratio; absence mucron and a value in males. From the results the isolate was Steinernema species in the feltiae-glaseri group of EPNs. Molecular examinations and further morphological investigations using scanning electron microscope are necessary to establish the actual identity, correct placement and phylogenic relationship of isolate TK1 with other Steinernematids.

Keywords: Entomopathogenic nematode, TK1, Morphology, morphometrics, Steinernema, feltiae-glaseri group

1. INTRODUCTION

Steinernematid and heteroharbditid nematodes are lethal to economically important agricultural pests (Shapiro-Ilan et al., 2002; Puza et al 2015). These nematodes infect and kill host within 48-

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72 hours and are safe to human and environment. Occurrence of EPNs has been reported worldwide but their biological potential has not been fully realized hence the need for search and identification of potent species (Hominick et al., 2002; Nguyen et al., 2007; Kalia, et al., 2014; Tumialis et al., 2016).

The EPNs exhibit differences in host range, infectivity, reproductive potential, commercial production and formulation. According to Puza et al., (2015), about 90 steinernematid species have been described worldwide while 15 species are from Africa. Finding and identifying indigenous EPNs population and species adapted to local climatic conditions is believed to be a crucial step for effective biological pest management an integral part of IPM (Salvadori et al., 2012; Kalia et al., 2014; Lima de Brida et al., 2017). Majority of EPN species have not been described. However, tools for their identification have been developed like the body features and DNA sequence linked to morphological traits (Thomas et al., 1997).

Morphometric and morphological data commonly based on male tail, size and shape of spicules, body size, presence or absence of mucron and lateral lines of infective juveniles are crucial for preliminary grouping of EPNs (Nguyen, 2007; Hating et al., 2009). The aim of the study was to identify unknown EPN Isolate TK1 on basis of its morphometric and morphological features.

2 MATERIALS AND METHOD

Nematode isolation and culturing

The nematode was isolated from soils in an avocado orchard at Kenya Agricultural and Livestock Research (KALRO) Kandara (Latitude -0◦ 99.65′ South, Longitude 37◦ 07.54′ East and altitude 1553m above the sea level). The nematodes were reared using insect baiting method as described by Bedding and Akhurst, (1975); Nguyen et al., (2007), where 250gms of soil was collected and 15 pre pupa stage of greater wax moth (Galleria mellonella) placed on the soil in a bowl. The samples were stored at room temperature of 25±20C and inspected for larval mortality every 24hr. The G. mellonella) cadavers were collected and examined for colour and presence of nematodes. Nematode culture named as TK1 isolate was established from infective juveniles (IJs) harvested using modified White trap method (White 1927).

Temporary and permanent specimen preparation

Cadavers of G. mellonella infected with EPN Isolate TK1 were surface sterilized in 70% ethanol, rinsed in distilled water and transferred into Ringer's solution where they were dissected. The required 20 fresh live 3rd stage IJs of isolate TK1 were individually picked under dissecting microscope as they emerged from cadavers 5-7 day after infection. Also, a total of 20 live males of isolate TK1 were obtained from infected G. mellanolla cadaver 3 days after baiting.

The nematodes were heat killed in a water bath at 50-60 ˚C for 3min. The specimens were fixed in 2-3 drops of double strength TAF fixative (14ml of 40%, of formaldehyde + 4ml triethanolamine in 82ml distilled water) (Courtney et al., 1955). After 48h temporary mounts that were used within a week were prepared. Permanent mounts were made according to Seinhorst, 1959; Ryss, 2017). The fixed nematodes were placed in a excavated glass block and 7ml of

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Solution 1 (20parts of 95% ethanol and 1part of glycerine in 79parts of distilled water) added. The glass block with samples was slowly dried by evaporation in a desiccator stuffed with cotton soaked in 95% alcohol. The glass block was placed in an oven at 35˚C for 12hours after which it was flooded with Solution 2 (95parts of 95% ethanol and 5parts of glycerin); put in the oven at 40˚C for 4hour after which the nematodes were mounted on microscope slides. The cover slip was supported by glass beads and sealed with wax or nail vanish. Morphological features considered for morphometrics were, nematode body length (L), maximum body diameter (MBD), anal body diameter (ABD), length tail (TL), distance from anterior end to oesophagus/pharynx (ES), spun from anterior end to excretory pore (EP), Hyaline (H) and in males spicule length (SL), gubernaculum length (GL), presence and absence of mucron was considered (Nguyen and Smart,1995; Nguyen, 2007; Dolinski et al., 2008; Mwaniki et al., 2009; Ikoyi, 2012; Nguyen, 2017). Data on morphometric was collected and expressed in micrometers. Photographs or images showing various nematode features were taken using compound microscope Leica Suit, DM 750 (LAS EZ Version 3.3.0 Build.181), fitted with digital color microscope camera LEICA EC4 (Leica Microsystems Switzerland Ltd).

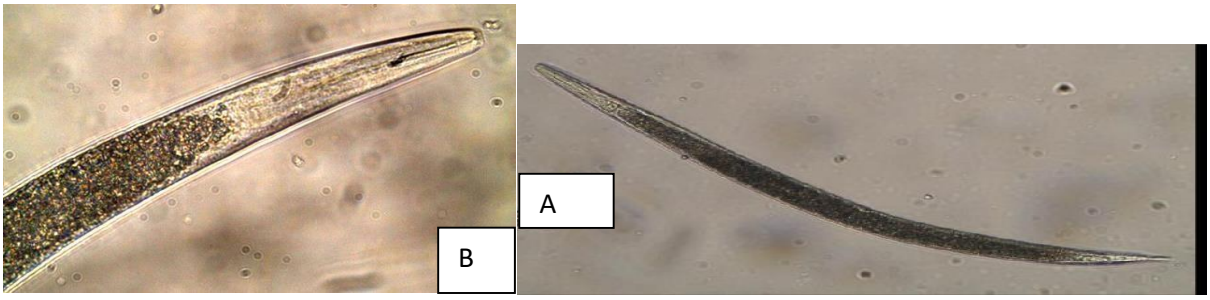
3. DATA ANALYSIS

The morphometric data was analysed using Excel 2013 version, means and ratios calculated. The results were also compared to the existing information of described nematodes.

4. RESULTS

4.1 Morphology of Third stage infective juveniles of EPN isolate TK1

Tan/light brown colour was observed in G. mellonella cadavers infected with isolate TK1. The morphometric data for the 3rd stage IJs and 1st generation males of isolate TK1 are summarized in Table 1. The IJs had an elongate body (834.54±87.36µm) length twice that of males (1781.33±195.07µm) while maximum body diameter (MBD) was two times and half bigger than the males MBD (113.46±20.87µm). Heat killed juveniles were either straight or slightly C-shaped. The IJs body tapered gradually anteriorly and posteriorly. The anterior end was blunt. The IJs had a short hyaline 20.47±3.33 (45-24.95) µm that was half their tail length (Plate 1 A, B, C, and Table 1).



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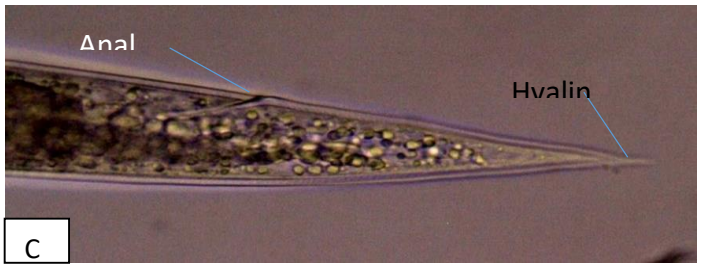

Plate 1. Infective juveniles of EPN Isolate TK1: (A) Slightly C shape, (B) Tapered but blunt ended anterior terminus and (C) Tail region with anal pore and hyaline

4.2. Morphology of first generation males of EPN Isolate TK1

The heat killed males assumed a J shape, tapered anteriorly, posterior end strongly ventrally curved and average body length of 1781.33±195.07µm. The anterior terminus was flat and pharynx more or less cylindrical. Procorpus and metacorpus cylindrical and same width but the isthmus was indistinct. The excretory pore was 106.37±13.77µm more posterior to the mouth, 2/3 of pharynx region and anterior to basal bulb. The basal bulb was rounded with a narrow base. The spicule was 82.05±13.94µm long, large with rounded anterior end, strongly curved, paired and golden brown in colour. It was slightly curved, the head (manubrium) wider than long, short shaft (calomus) present, velum wide and the spicule tip conoid. The gubernaculum was about half (45.28±5.89 µm) the spicule length and curved ventrally. The tail was short, an average of 19.97±2.84µm long, rounded blunt end and mucron absent and slightly longer than half anal body diameter (Plate 5.2 A, B and C; Table 5.1). The male had 3 pairs of subterminal postcloaca papillae, single pappilae, 5 sub ventral and adanal pair posterior to cloaca. Testis reflexed ventrally and monarchic.

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Plate 2: First generation males of EPN Isolate TK1: (A) posterior strongly J shaped male, anterior region with excretory pore and (C) Tail region with spicule and gubernaculum

Table 1. Morphometrics of 3rd stage Infective juveniles and 1st generation males of EPN isolate TK1

Character	Infective juveniles	First generation males
n	20	20
L	834.54±87.36 (658.60-986.89)	1781.33±195.07 1296.62-2096.87
a	17.79±1.06 (16.26 -19.70)	15.98± 2.22 12.43-19.45
b	7.98±1.57 (5.90 - 12.15)	15.07±2.19 (10.76-19.44)
c	15.94±2.30 (11.86 - 20.45)	91.35±18.60 (57.17-125.56)
c′	2.45±0.33 (1.82 - 2.98)	- -
EP	81.23±7.06 (62.66-95.57)	106.37±13.77 84.51-140.13
MBD	46.92±4.23 (38.98- 55.30)	113.46±20.87 (90.21-162.74)
ES	106.57±12.78 (70.98-118.86)	119.39± 13.57 (93.10-150.77)
T	53.22±8.35 (40.30-71.51)	19.97±2.84 (14.20-25.70)
ABD	21.85±2.83 (16.50-25.97)	33.23 ±4.87 (25.62-45.03)
H	20.47±3.33 (13.45-24.95)	- -
SL	- -	82.05±13.94 (57.86-128.30)
GL	- -	45.28±5.89 (34.48-54.87)

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SW%	-	73.60±13.01 (50.90-89.53)
GS%	-	56.30±10.32 (36.98-86.28)
	-	
D%	77.59±14.00 (61.80-114.09)	89.18± 6.49 (77.70-100.78)
E%	291.22±34.69 (219.02-363.80)	542.72±105.13 (424.35-785.40)
H%	2.47±0.45 (1.65-3.37)	- -

n=No of specimen; a=L/MBD; b=L/ES; c=L/T; c′=T/ABD; D%=EP/ES*100; E%EP/T*100; GS%=GL/SL*100; SW%=SL/ABD*100; H%=H/TL*100; L= body length; MBD=maximum body diameter, ABD=anal body diameter; T=length tail; ES=pharynx length; EP=excretory pore; SL; spicule length, GL=gubernaculum length; -, Data not available

Comparative morphometrics of infective juveniles of EPN isolate TK1

The average body length (834.54±87.36 µm) was close to S. ethiopiense (898±50 µm). The b ratio and maximum body diameter (MBD) compared narrowly with S. jeffreyense, S. ethiopiense and S. pwaniensis. The c ratio of Isolate TK1 (15.94±2.30 µm) was very close to all the comparator juveniles. The excretory pore (81.23±7.06 µm) of isolate TK1differed distantly from S. karii (74±3 µm) but was close to S. jeffreyense, S. ethiopiense and S. pwaniensis. The tail (53.22±8.35 µm) and hyaline (20.47±3.33 µm) were far too short (compared to the S. jeffreyense, S. ethiopiense and S. pwaniensis (Table 2).

Table 2. Comparative morphometrics of infective juveniles of EPN isolate TK1 with The Means±SD and ranges in µm

	Fresh Infective juveniles				
Character	TK1	*S. jeffreyense*[1]	*S.karii*[2]	*S.ethiopiense*[3]	*S.pwaniensis*[4]
n	20	20	20	20	20
L	834.54±87.36 (658.60-986.89)	926±83 (784–1043)	932±24 (876-982)	898±50 (768-1010)	978±75 (808-1131)
a	17.79±1.06 (16.26 -19.70)	26 ±3.2 20-35	28.5 -	27±2.6 (20-30)	25±1.4 (23-29)
b	7.98±1.57 (5.90 - 12.15)	7.0± 0.5 (6.1-7.9)	- -	6.4±0.3 (5.7-7.1)	7.0±0.4 (6.2-7.6)
c	15.94±2.30 (11.86 - 20.45)	12± 22.0 (9.7-19)	12.6 -	12±0.6 (11-14)	11±0.7 (10-12)
c′	2.45±0.33 (1.82 - 2.98)	2.7± 0.4 (2.1-3.5)	- 3.4	3.2±0.2 (2.9-3.8)	3.8±0.5 (3.3-5.4)
EP	81.23±7.06 (62.66-95.57)	87± 9.3 (78-107)	74±3 (68-80)	78±4.6 (65-84)	86±4.7 (80-95)
MBD	46.92±4.23	35 ± 4.4	33±1.4	34±3.0	39±3.1

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	38.98- 55.30)	(23- 43)	(31-35)	(32-43)	(32-45)
ES	106.57±12.78	133±7.2	-	140±6.9	140±5.7
	(70.98-118.86)	(116-149)	-	(127-153)	(130-151)
T	53.22±8.35	81±10.1	74±4.5	73±3.9	87±5.4
	(40.30-71.51)	(50-96)	(64-80)	(64-80)	(75-95)
ABD	21.85±2.83	39±4.6	22±0.6	23±1.3	23±2.2
	(16.50-25.97)	(19-39)	(64-80)	(19-24)	(17-27)
H	20.47±3.33	44±6.3	-	38±3.7	46±4.7
	(13.45-24.95)	20-52	-	(29-43)	(37-54)
D%	77.59±14.00	66±6.1	-	56±2.2	61±2.6
	(61.80-114.09)	57-85	-	(51-58)	(57-67)
E%	291.22±34.69	109± 19	96	107±5.5	98±6.4
	(219.02-363.80)	(86-169)	-	(91-116)	(83-110)
H%	2.47±0.45	43±6.3	-	52±3.0	53±3.1
	(1.65-3.37)	(20-52)		(43-56	(49-60)

n=No of specimen; a=L/MBD; b=L/ES; c=L/T; c′=T/ABD; D%=EP/ES*100; E%EP/T*100; GS%=GL/SL*100; SW%=SL/ABD*100; H%=H/TL*100; L= body length; MBD=maximum body diameter; ABD=anal body diameter; T=length tail; ES=pharynx length; EP=excretory pore; SL=spicule length; GL=gubernaculum length; H=Hyaline; Data from: 1Malan, et al., 2015; 2Waturu et al., 1997; 3Tamaru, et al., 2012; 4Puza et al., 2015; -Data not available; Data in highlight, indicate close means

Comparative morphometrics of first generation males of isolate TK1

The body length, anterior distance to excretory pore, and spicule length (1296.62-2096.87, 106.37±13.77µm and 82.05±13.94 µm) of TK1 were close to that of S. karii, (1400-2400, 108±14 and 83±4 µm). Ratios a= L/MBD and b=L/ES (15.98± 2.22; 15.07±2.19 µm) of isolate TK1 measured closely to S. jeffreyense (12± 1.7; 11±1.0µm) and ratio b only with S. pwaniensis (12±1.3µm). The gubernaculum length (45.28±5.89µm) compared narrowly with S. ethiopense (49±1.3). The D%=EP/ES*100 was close (89.18±6.49µm) to S. pwaniensis (85±8.0 µm). The Isolate TK1 tail and anterior distance to excretory pore length varied distantly from the comparison species but a mucron was absent in all (Table5.3).

Table 3. Comparative morphometrics of first generation males of EPN isolate TK1 with mean ±SD and ranges in µm

Character	First generation males				
	TK1	S. jeffreyense[1]	S. karii[2]	S. ethiopense[3]	S. pwaniensis[4]
n	20	20	20	20	20
L	1781.33±195.07	1634±130	*1900±0.3	1081±40	2104±247
	1296.62-2096.87	(1740–1899)	1400-2400	(1028-1232)	(1616-2586)
a	15.98± 2.22	12± 1.7	*13.7	12±0.6	13±1.7
	(12.43-19.45)	(9.9 –15.2)	(11.7-17.1)	(11-13)	(11-16)
b	15.07±2.19	11±1.0	*11.3	7.1±0.2	12±1.3
	(10.76-19.44)	(9.4-13)	(9.0-13.3)	6.3-7.4	(9.6-15)

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c	91.35±18.60 (57.17-125.56)	61±6.2 (49-72.5)	*50 (42-70)	42±3.4 (35-49)	50±6.2 (38-66)
EP	106.37±13.77 84.51-140.13	71± 10 (50-99)	108±14 (86-138)	88±1.7 85-92)	145±16 (113-172)
MBD	113.46±20.87 (90.21-162.74)	139±22 (94-167)	*136±17 (107-166)	92±48 (81-99)	158± 26 (109-196)
ES	119.39± 13.57 (93.10-150.77)	151±8.5 (136-165)	164±7 (146-187)	153±3.1 (149-161)	170±11 (152-192)
T	19.97±2.84 (14.20-25.70)	27±3.01 (21-33)	37±7.5 (22-48)	29±1.9 (25-33)	42±5.0 (30-56)
ABD	33.23 ±4.87 (25.62-45.03)	42±3.8 (35-49)	55±5 (43-66)	45±0.8 (43-46)	53±5.8 (43-65)
SL	82.05±13.94 (57.86-128.30)	88±3.6 (79-95)	83±4 (73-91)	73±2.0 (69-77)	92±4.6 (80-97)
GL	45.28±5.89 (34.48-54.87)	57±2.8 (51-61)	57±6 (42-64)	49±1.3 (46-57)	60±2.2 (56-64)
SW%	73.60±13.01 (50.90-89.53)	215±27 (171-295)	*151 -	164±5.8 154-175	178±19 (146-226)
GS%	56.30±10.32 (36.98-86.28)	65±2.57 (61-71)	*70±1 (50-80)	67±2.1 (63-70)	65±2.9 (61-72)
D%	89.18± 6.49 (77.70-100.78)	47±8.4 (34-68)	66 (57-78)	57±1.6 (54-61)	85±8.0 (71-98)
E%	542.72±105.13 (424.35-785.40)	47±8.4 (34-68)	*240 -	304±23 (264-357)	345± 51 (274-453)
Mucron	Absent	Absent	Absent	Absent	Absent

n=No of specimen; a=L/MBD; b=L/ES; c=L/T; D%=EP/ES*100; E%=EP/T*100; GS%=GL/SL*100; SW%=SL/ABD*100; L= body length; MBD=maximum body diameter; ABD=anal body diameter; T=length tail; ES=pharynx length; EP=excretory pore; SL=spicule length, GL=gubernaculum length

1Malan, et al., 2015; 2Waturu et al., 1997; 3Tamaru, et al., 2012; 4Puza et al., 2015; *after Nguyen and Hunt, 2006 Data in highlight, indicate close means; -, Data not available

5 DISCUSSIONS

Tan/light brown colour was observed in G. mellonella cadavers infected with isolate TK1. This suggested that the EPN isolate belongs to genus Steinernema. Shades of brown colour are reported in G. mellonella infected with EPNs Steinernema species. The characteristic features in IJs of isolate TK1 was body length of 834.54±87.36 (658.60-986.89) µm; end distance to excretory pore 81.23±7.06 (62.66-95.57) µm; short tail length 53.22±8.35 (40.30-71.51) µm and short hyaline (20.47±3.33 (13.45-24.95) µm. First generation males were characterized by body length of 1900±0.3 (1296.62-2096.87) µm; lack of a mucron on the tail tip; spicule length 82.05±13.94(40.30-71.51) µm; light golden brown colour of the spicule; short gubernaculum 45.28±5.89µm; wide than long spicule head with short shaft. The tail was short, an average of 19.97±2.84µm long equivalent to IJs hyaline length. The heat killed males were J shaped but strongly curved posteriorly almost spiral.

Based on body length of IJs, Steinernema species are grouped into glaseri (>1000µm), feltiae (<1000 but >700µm), intermediate (<700 but >600µm), carpocapsae (<600µm) and bicornutum (with 2 horn like structures) group (Nguyen, 2017). Thus by morphometrics isolate TK1 belongs

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to "feltae" group as the average body length of IJs was 834.54±87.36 (ranges 658.60-986.89) µm. The comparative species S. jeffreyense, S. karii and S. ethiopense and S. pwaniensis IJs body length 926±83,932±24, and 978±75µm respectively also fits feltae group. The IJs of isolate TK1 were distinct in that the pharynx length was short 106.57±12.78 (70.98-118.86) µm; tail 53.22±8.35 (40.30-71.51) µm; high E% of 291.22±34.69 (219.02-363.80) µm; short pharynx length 106.57±12.78 (70.98-118.86) µm. The males isolate is separated from the comparators in a number of characteristics which include golden brown spicule; very short tail in males19.97±2.84 (14.20-25.70) µm; large c ratio 91.35±18.60 (57.17-125.56) µm; large E% value 542.72±105.13(424.35-785.40) µm; short pharynx 119.39± 13.57 (93.10-150.77) µm. Thus isolate is closely related morphometrically and morphologically to S. karii, S. pwaniensis, S. ethiopense and S. jeffreynse which are geographically Africa in origin.

6 CONCLUSIONS

The isolate TK1 is a Steinernematidae due to the tan/light brown colour observed in nematode TK1 infected G. mellonella cadavers. It also had some similar morphometric and morphology with S. karii, S. pwaniensis, S. ethiopense and S. jeffreynse which are Steinernema species and African in origin. The isolate is placed in feltiae-glaseri group. It was distinct from the other three described species by its IJs and males, short pharynx tail and hyaline and golden light brown colour of the spicule of EPNs and strong ventral posterior curvature of heat killed males. The isolate is therefore suspected to be a new Steinernema species from Kenya, however further morphological investigations using Scanning Electron Microscope (SEM) and also molecular techniques are recommended to ascertain the identity of the isolate.

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